THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 51, Issue of December 21, pp. 48292–48299, 2001
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Printed in U.S.A.

The Cyclin-dependent Kinases cdk2 and cdk5 Act by a Random, Anticooperative Kinetic Mechanism*

Received for publication, March, 2001, and in revised form, September 5, 2001
Published, JBC Papers in Press, October 16, 2001, DOI 10.1074/jbc.M102034200

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cdk2·cyclin E and cdk5·p25 are two members of the cyclin-dependent kinase family that are potential therapeutic targets for oncology and Alzheimer's disease, respectively. In this study we have investigated the mechanism for these enzymes. Kinases catalyze the transfer of phosphate from ATP to a protein acceptor, thus utilizing two substrates, ATP and the target protein. For a two-substrate reaction, possible kinetic mechanisms include: ping-pong, sequential random, or sequential ordered. To determine the kinetic mechanism of cdk2·GST-cyclin E and cdk5·GST-p25, kinase activity was measured in experiments in which concentrations of peptide and ATP substrates were varied in the presence of dead-end inhibitors. A peptide identical to the peptide substrate, but with a substitution of valine for the phosphoacceptor threonine, competed with substrate with a K_i value of 0.6 mM. An aminopyrimidine, PNU 112455A, was identified in a screen for inhibitors of cdk2. Nonlinear least squares and Lineweaver-Burk analyses demonstrated that the inhibitor PNU 112455A was competitive with ATP with a K_i value of 2 μM. In addition, a co-crystal of PNU 112455A with cdk2 showed that the inhibitor binds in the ATP binding pocket of the enzyme. Analysis of the inhibitor data demonstrated that both kinases use a sequential random mechanism, in which either ATP or peptide may bind first to the enzyme active site. For both kinases, the binding of the second substrate was shown to be anticooperative, in that the binding of the first substrate decreases the affinity of the second substrate. For cdk2·GST-cyclin E the kinetic parameters were determined to be $K_{m,\,ATP}$ = 3.6 ± 1.0 μM, $K_{m,\,peptide}$ = 4.6 ± 1.4 μM, and the anticooperativity factor, α = 130 ± 44. For cdk5·GST-p25, the $K_{m,\,ATP}$ = 3.2 ± 0.7 μM, $K_{m,\,peptide}$ = 1.6 ± 0.3 μM, and α = 7.2 ± 1.8.

Kinases are a major component of the signal transduction pathways involved in cellular regulation. In addition to their role in maintaining normal homeostasis, there is increasing evidence implicating these enzymes in various diseases, such as cancer, neurodegeneration, and inflammation. Increased levels of enzymatic activity can lead to pathway deregulation, as exemplified by a number of oncogenic kinases, including Akt, Src, and Raf. The important role of kinases in health and disease has led to the suggestion that kinases may be good therapeutic targets (for review, see Refs. 1, 2). In fact, inhibitors of several kinases, such as protein kinase C and p38 MAPK,[1] are in clinical development as cancer and inflammation therapeutics, respectively (3, 4). Two members of the cyclin-dependent kinase family, cdk2 and cdk5, have been implicated in cancer and Alzheimer's disease, respectively, and inhibitors of these kinases may prove to be clinically useful.

cdk2 is a member of the cyclin-dependent kinase family, which binds cyclins A and E and regulates cell cycle progression (for review, see Refs. 5, 6). Disruption of the normal cell cycle is a hallmark of cancer, and deregulation of the cyclin·cdk complexes is associated with the disease (7, 8). For example, increased cdk2 kinase activity was detected in tumor tissues in a mouse mammary tumor model. In addition, cyclin E protein levels were increased in the tumor tissue, and a number of variant cyclin E isoforms also were detected in tumor, but not in normal tissues (9). In human tissues, cyclin E levels have been shown to be increased in some breast, colon, and leukemic cancers. The inhibitory proteins p21, p27, and p16 are deleted or mutated in some tumor types, further supporting the idea that deregulation of cdk activity may contribute to oncogenesis (10–12). Taken together, these results have led to the hypothesis that the cell cycle checkpoints are good points for therapeutic intervention. In fact, a number of cdk inhibitors currently are in phase I and phase II development as cancer therapeutics (13–15).

cdk5 is a unique member of the cdk family of kinases involved in neuronal function (for review, see Refs. 4, 16). Although the cdk5 protein is widely expressed in many tissues and cells, cdk5 kinase activity is restricted to neuronal cells. This tissue specificity is the result of the cdk5 activator proteins (p35, p25 (an N-terminally truncated form of p35), and p39), which are expressed only in brain (17–20). Results from a number of studies, including dominant negative mutant forms of cdk5 and knock-out mice, demonstrate that the cdk5·p35 complex plays an essential role in neurite outgrowth and neuronal differentiation (21, 22).

Increased cdk5·p35 kinase activity has been implicated in

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The atomic coordinates and structure factors (code 1JSV) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; MEK, MAPK/extracellular signal-regulated kinase kinase; cdk, cyclin-dependent kinase; GST, glutathione *S*-transferase; AD, Alzheimer's disease; NFT, neurofibrillary tangles; Rb, retinoblastoma; AIC, Akaike's information criterion; EGF, epidermal growth factor; PEST, proline (P), glutamic acid (E), serine (S), threonine (T); PKV, PKVPKKAKKL peptide.

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Alzheimer's disease. Hyperphosphorylated tau protein is the major component of the neurofibrillary tangles (NFTs) found in Alzheimer's disease (AD) brain, and *in vitro* experiments have demonstrated that cdk5·p35 phosphorylates sites on tau that are also phosphorylated on NFT tau (23). Immunocytochemical experiments have shown that cdk5 co-localizes with NFT-tau in pretangle neurons (24), and cdk5 enzyme activity in AD brain is increased ~2-fold compared with tissue from age-matched controls (25). Neurons from the brain tissue of AD patients have increased levels of p25, the truncated form of p35, and the p25·cdk5 complex shows increased tau phosphorylation, compared with the p35·cdk5 complex (26). Cell death induced by amyloid β-peptide was inhibited both by a cdk inhibitor and a calpain inhibitor, suggesting that abnormal accumulation of p25 may contribute to cell death and AD pathology (25).

The role of cdk2 and cdk5 in proliferation and neuronal function has led to the idea that these kinases may be good therapeutic targets (27–29). However, a detailed understanding of the kinetic mechanisms by which these enzymes act is an important step in the rational design of inhibitors, but it has not been previously reported. A number of studies have been carried out identifying the kinetic mechanisms of various kinases (30–32). The results of these studies have been varied, and no one mechanism has emerged for all kinases. The majority of the kinases appear to act via a sequential and not a ping-pong mechanistic pathway, although both sequential random and sequential ordered mechanisms have been reported. In this study we have determined the mechanistic pathway for cdk2·cyclin E and cdk5·p25. As with other kinases, these enzymes utilize two substrates, ATP and protein (peptide). We show that both enzymes act via a random sequential mechanism, and furthermore, that the two substrates bind in an anticooperative fashion.

EXPERIMENTAL PROCEDURES

Enzyme Purification—High-Five insect cells were co-infected with cdk5 and GST-p25 or cdk2 and GST-cyclin E and harvested after 66 h. The cell pellets were solubilized in 20 mM HEPES, pH 7.3, containing 20 mM NaCl, 1 mM EDTA, 2 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A. The solutions were taken through one cycle of freeze-thaw, followed by homogenization with a Dounce homogenizer. The homogenates were centrifuged at 39,000 × *g* for 60 min, and the supernatant liquid was decanted and filtered through a Nalgene 0.2-μm filter to remove particulates. A column (1.0-ml bed volume) was packed with glutathione-Sepharose (Amersham Biosciences, Inc.) and equilibrated with 20 mM HEPES, pH 7.3, containing 150 mM NaCl. The filtered supernatant was applied to the column at a rate of 12 ml/h. After loading, the column was washed with 30 ml of equilibration buffer. The bound protein was eluted at a rate of 12 ml/h with 50 mM Tris/HCl, pH 8.0, containing 10 mM reduced glutathione. Pools from column chromatography were subjected to analysis by SDS-PAGE and protein determination prior to use in kinase assays. Small amounts of purified untagged cdk5·p25 and cdk2·cyclin E complexes were purified from infected High-Five insect cells and initially used to compare with the tagged complexes. No differences were observed between the complexes in the kinetics, or in inhibition profiles, and thus due to the ease of large scale purification, the GST-tagged complexes routinely were used in the majority of studies.

Kinase Assays—Kinase assays were carried out in buffer containing 50 mM HEPES, 15 mM $MgCl_2$, 1 mM dithiothreitol, 20 μM Na_3VO_4, 0.1 mg/ml bovine serum albumin, unlabeled ATP, and peptide substrate (histone H1-derived peptide PKTPKKAKKL). The sequence of the peptide inhibitor (referred to as PKV) is PKVPKKAKKL. In experiments utilizing PKV, the HEPES concentration was 100 mM. Reactions were carried out in duplicate in a 50-μl volume containing 2 μCi of [γ-^{33}P]ATP and 0.5 nM cdk5·GST-p25 or 23 nM cdk2·GST-cyclin E-ΔPEST (referred to as cdk2·GST-cyclin E). Cyclin E-ΔPEST is a mutant version of cyclin E containing an altered PEST degradation sequence (33). This mutant is hyperstable *in vivo*, because the proteolytic degradation targeting motif is mutated. The PEST sequence is found at the C-terminal end of cyclin E. Wild-type cyclin E has the sequence RASPLPSGLLTPPQSGK, and the mutant cyclin E-ΔPEST contains the sequence RASPLPSGLLIAAQGGK. Unlabeled ATP and peptide substrate were used at varying concentrations as indicated. The reactions were carried out at 37 °C for 20 min. Twenty-microliter aliquots were spotted on Whatman P81 phosphocellulose paper, washed three times in 1% phosphoric acid, dried, and counted. Total picomoles of phosphate incorporated were calculated for each sample.

Crystallography—cdk2 was purified from infected High-Five insect cells following the protocols outlined by Rosenblatt *et al.* (34). Protein materials having a concentration of 0.5–1.0 mg/ml, 1 mM EDTA, 20 mM HEPES, pH 7.4 were quick-frozen with liquid nitrogen and stored at −80 °C. For crystallization, this material was thawed and filtered through a 0.22-μm filter. The material was then concentrated using a centrifuge using10K Centriprep tubes at 3000 × *g* and a temperature of 5 °C. Protein concentrations of around 3 mg/ml were used in the crystallization setups. Trays of hanging drops were setup at 5 °C over a well solution of 200 mM HEPES, pH 7.4, and 0.1% β-mercaptoethanol. Crystals larger than 0.5 mm were grown that diffracted above 1.5-Å resolution on synchrotron beamlines. The inhibitor, PNU 112455A, was dissolved in Me_2SO and added to the hanging drop of the well solution containing a crystal of cdk2.

Diffraction data of the cdk2·PNU 112455A complex were collected on a Siemens Hi-Star area detector/rotating anode x-ray generator system using CuK_α radiation at an approximate temperature of 100 K. Data were processed and reduced to integrated intensities using the Siemens software, SADIE and SAINT. The crystals belong to space group $P2_12_12_1$ with cell dimensions of $a = 54.00$ Å, $b = 72.1$ Å, $c = 72.2$ Å. The R_{merge} (below) was 0.049 for the 18,495 intensities to a resolution of 1.96 Å with a completeness of 91.4% and a redundancy of 3.88. For the shell from 2.03 to 1.96 Å, the R_{merge} was 0.18 for the 34% of the intensities greater than 2σ. The atomic coordinates of the cdk2·ATP complex supplied in Ref. 35 were used as the initial model. The structural model was refined ($R_F = 0.162$ for 16,429 reflections $> 2\sigma_F$ and 0.185 for 18,454 all reflections) using XL from the SHELX-97 software system (36). Residues 36–46 could not be seen in the electron density maps and were not included in the model used in the refinement. The electron densities for all the non-hydrogen atoms of the inhibitor were very clearly seen in the electron density maps. Standard deviations of covalent bond lengths and bond angles from ideality were 0.013 Å and 1.9°, respectively. Procheck (37) indicates that all parameters are inside acceptable limits. Additional data collection statistics, refinement statistics, and atomic coordinates are deposited in the Protein Data Bank (entry 1JSV).

$$R_F = \frac{\sum_{hkl} |F_o - F_c|}{F_{hkl}} \quad \text{(Eq. 1)}$$

$$R_{merge} = \frac{\sum_{hkl} \sum_{i=1}^{N} |\langle I^{hkl} \rangle - I_i^{hkl}|}{\sum_{hkl} \sum_{i=1}^{N} I_i^{hkl}} \quad \text{(Eq. 2)}$$

Where N is the number of symmetry-related reflections.

Data Analysis—Data were analyzed by the nonlinear least squares method, using software described by Yamaoka *et al.* (38), and commercial software, GraFit version 4.03 (Erithacus Software). The kinetic pathways and corresponding velocity equations are shown below.

Ping-pong (double displacement) mechanism,

$$v = \frac{k_{cat}[E_0][A][B]}{K_a[B] + K_b[A] + [A][B]} \quad \text{(Eq. 3)}$$

Rapid equilibrium ordered,

$$v = \frac{k_{cat}[E_0][A][B]}{K_a \cdot K_b + K_b[A] + [A][B]} \quad \text{(Eq. 4)}$$

Rapid equilibrium random,

$$v = \frac{k_{cat}[E_0][A][B]}{\alpha \cdot K_a \cdot K_b + \alpha \cdot K_a[B] + \alpha \cdot K_b[A] + [A][B]} \quad \text{(Eq. 5)}$$

Replot equations for random mechanism,

$$\frac{1}{V_{max}(app)} = \frac{\alpha K_a}{V_{max}} \frac{1}{[A]} + \frac{1}{V_{max}} \quad \text{(Eq. 6)}$$

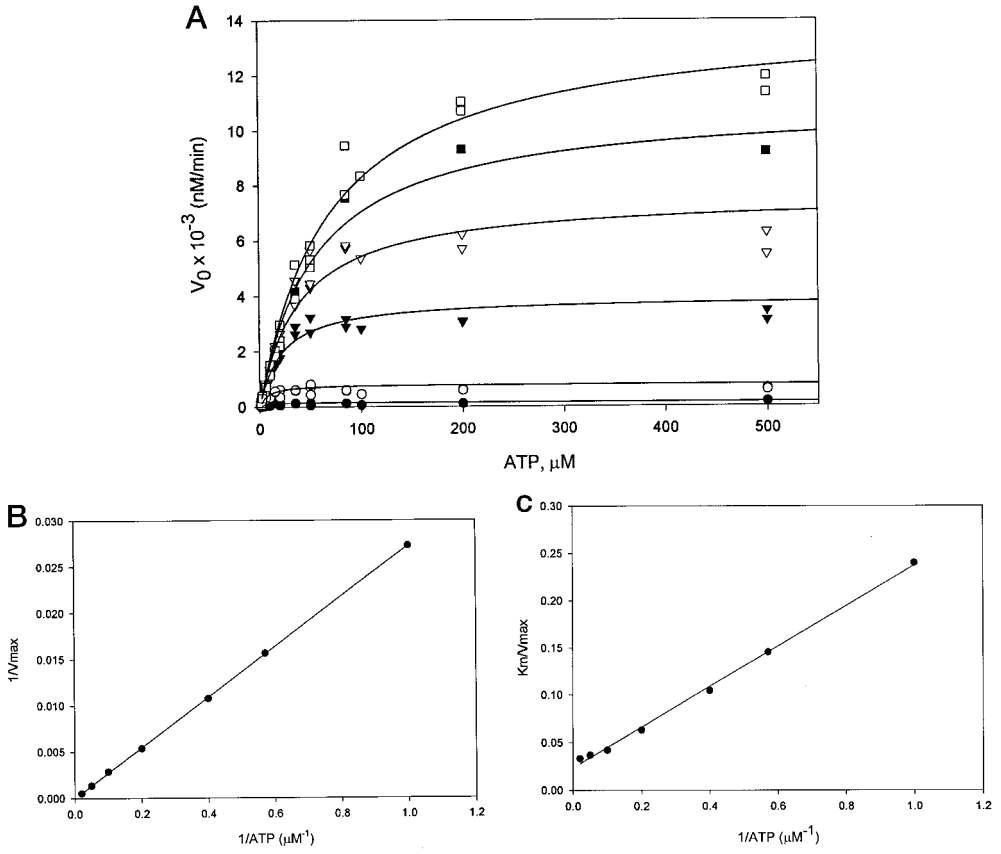

FIG. 1. **Data plots for cdk2·GST-cyclin E.** *A*, Michaelis-Menten plot of velocity *versus* ATP in assay. Five data sets were combined and normalized to generate this plot. The data from each set are shown, and the lines are from the combined data are fitted to the rapid equilibrium random/steady-state ordered model. Peptide concentrations: □, 100 μM; ■, 75 μM; ▽, 50 μM; ▼, 25 μM; ○, 5 μM; ●, 1 μM. *B* and *C*, replot analysis showing data from one data set.

$$\frac{K_m(\text{app})}{V_{\max}(\text{app})} = \frac{\alpha K_b K_a}{V_{\max}} \frac{1}{[A]} + \frac{\alpha K_b}{V_{\max}} \qquad \text{(Eq. 7)}$$

RESULTS

The cdk2 and cdk5 substrate consensus sequence for phosphorylation is Ser/Thr-Pro-*X*-Arg/Lys (39, 40). A number of peptides have been shown to serve as substrates for cdk2 and cdk5, and a peptide derived from histone H1, PKTPKKAKKL, was chosen for use in these studies (41–43). This peptide contains only one amino acid available for phosphorylation, which is desirable for kinetic experiments because the presence of multiple phosphorylation sites complicates the interpretation of results. Using the histone-derived substrate peptide and purified complexes of cdk2·GST-cyclin E and cdk5·GST-p25, time course experiments at 37 °C were carried out to establish the linear range of the assays. All subsequent assays were conducted at 37 °C for 20 min, which was within the linear range for the chosen enzyme concentration (data not shown).

To determine the kinetic mechanism for the enzymes, replot analysis, as well as dead-end inhibitors were used. The two substrates, ATP and peptide, were varied within a single experiment, and the activities of cdk2·GST-cyclin E (Fig. 1*A*) and cdk5·GST-p25 (Fig. 2*A*) were measured. Each data set first was analyzed using equations describing ping-pong, random, and ordered mechanisms. The rapid equilibrium velocity equations describing these mechanisms are mathematically distinct with respect to their denominators (see "Experimental Procedures"), however, they cannot be used to discriminate between random and steady-state ordered pathways.

Nonlinear least squares analysis was used to determine the most probable pathway (Table I). In comparing the kinetic mechanisms, the most significant fit was determined through the use of sum of squares, the F test, and Akaike's information criterion (AIC) (38, 44–46). As shown by the very low sum of squares, 1.2, the random mechanism clearly gave the best fit to the data. However, because the velocity equation describing this mechanism contains one additional parameter as compared with the other pathways, the low sum of squares value alone is not sufficient as a basis for choosing the best fit model. The AIC is useful for distinguishing between models with differing numbers of parameters. In general, when comparing models with differing numbers of parameters the model with the least positive AIC is superior. The AIC for the random pathway ranged from 2- to 4-fold lower in the case of cdk2·GST-cyclin E and 4- to 8-fold lower in the case of cdk5·GST-p25, compared with the other pathways. On the basis of this comparison the random model was the most probable kinetic pathway for both cdk2·GST-cyclin E and cdk5·GST-p25. Finally, we applied an F test comparison of the random model to each of the other models. As can be seen by the very low p values, in each case less than 0.01, the random pathway kinetic model fits the data for both cdk2·GST-cyclin E and cdk5·GST-p25 significantly better than either the ordered or ping-pong models.

Replots of the data graphically demonstrate the mathematical differences between the velocity equations representing the three considered pathways. A plot of $K_m/V_{\max}$ *versus* 1/[ATP] will have a slope of zero for a ping-pong pathway and a positive slope for a sequential system (either ordered or random). A plot of $1/V_{\max}$ *versus* 1/[ATP] will have a slope of zero for a rapid equilibrium ordered pathway and a positive slope for a random or a ping-pong system. Replots of $K_m/V_{\max}$ *versus* 1/[ATP] and $1/V_{\max}$ *versus* 1/[ATP] for both cdk2·GST-cyclin E and

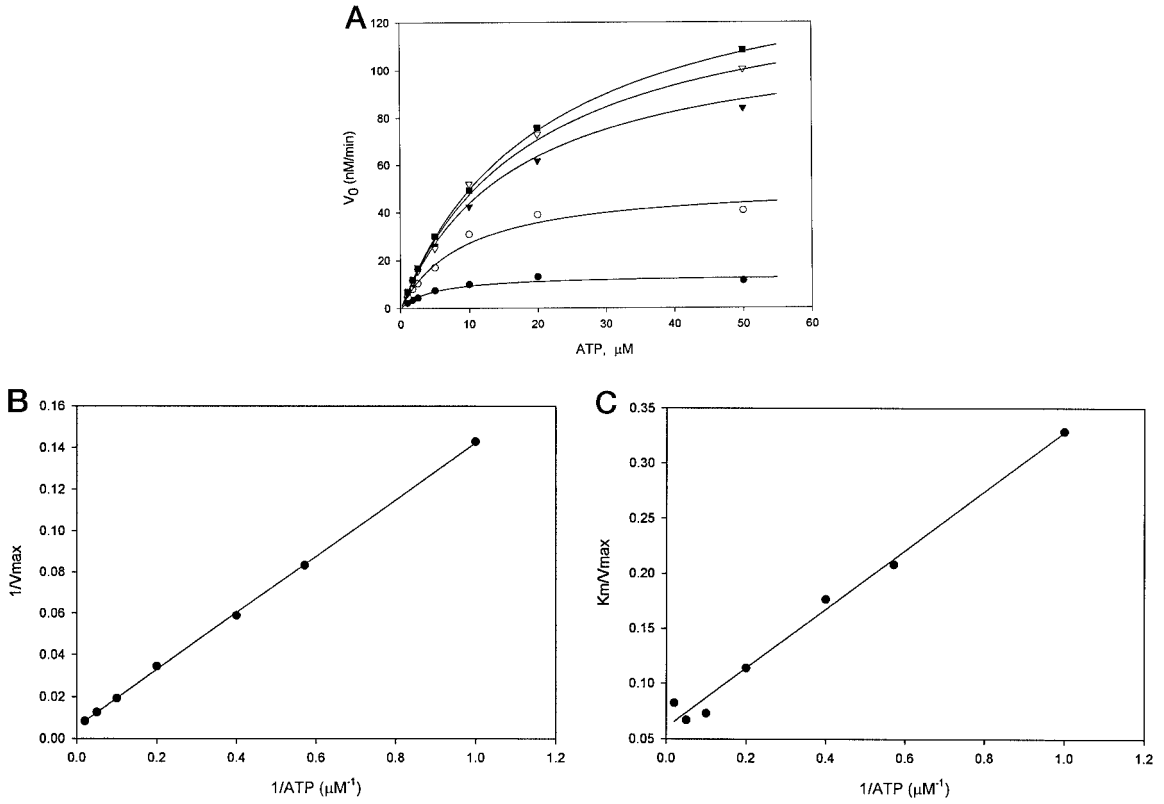

FIG. 2. **Data plots for cdk5·GST-p25.** *A*, Michaelis-Menten plot of velocity *versus* ATP in assay. One data set was used to generate this plot. A second experiment gave identical results. The *lines* are from the combined data fitted to the rapid equilibrium random/steady-state ordered model. Peptide concentrations: ■, 100 μM; ▽, 50 μM; ▼, 25 μM; ○, 5 μM; ●, 1 μM. *B* and *C*, replot analysis.

TABLE I
Summary of statistical analysis of kinetic mechanisms

Enzyme	Kinetic mechanism	Sum of squares	AIC	F test *p* value
Cdk2·cyclin E	Random or steady-state ordered	1.2	43	
	Rapid equilibrium ordered, ATP binds first	2.8	200	<0.01
	Rapid equilibrium ordered, peptide binds first	6.9	360	<0.01
	Ping-pong	2.6	190	<0.01
Cdk5·p25	Random or steady-state ordered	3.0	47	
	Rapid equilibrium ordered, ATP binds first	160	180	<0.01
	Rapid equilibrium ordered, peptide binds first	49	140	<0.01
	Ping-pong	9.0	83	<0.01

cdk5·GST-p25 all had positive slopes, which are consistent with both enzymes acting by a random ordered substrate binding mechanism (Figs. 1*B*, 1*C*, 2*B*, and 2*C*).

When rapid equilibrium conditions prevail, the initial velocity equations for ping-pong, random, and ordered mechanisms are mathematically distinct, and the results presented in Table I and Figs. 1 and 2 are sufficient for identifying the correct kinetic pathway. However, under a specific set of steady-state conditions the velocity equation describing an ordered mechanism becomes mathematically equivalent to that describing a random mechanism. For this to occur, the first order rate constant for the dissociation of the enzyme·ATP complex would have to be much smaller than the apparent k_{cat}. The apparent k_{cat} for a steady-state ordered mechanism where the concentration of products is zero is defined by the rate constant for the phosphotransferase step and all the rate constants for the enzyme·product dissociation steps. Although it seems unlikely that the dissociation of the enzyme·ATP complex would be significantly slower than the catalytic step and all subsequent product dissociation steps combined, this possibility cannot be ruled out on the basis of initial velocity data using enzymes and substrates alone.

To differentiate conclusively between a random *versus* steady-state ordered kinetic mechanism, competitive inhibitors for both substrates (ATP and peptide) were used (47). PNU 112455A is an aminopyrimidine (Fig. 3*A*) identified as a cdk2 inhibitor during screening of the Pharmacia compound collection. The K_i of this compound against cdk2·GST-cyclin E was 2.0 ± 0.2 μM (Fig. 3*B*), and for cdk5·GST-p25, it was 2.0 ± 0.3 μM (Fig. 3*C*). Kinase specificity testing was carried out on a limited basis and demonstrated that PNU 112455A showed some selectivity as a cdk inhibitor. When tested at 100 μM, PNU 112455A did not inhibit the c-Met or insulin-like growth-1 receptor tyrosine kinases, or cAMP-dependent kinase. The MAPK family, like the cdks, consists of proline-directed protein kinases. However, no inhibition of ERK2 activity was observed with 100 μM PNU 112455A (data not shown).

Experiments were carried out using PNU 112455A with both cdk2·GST-cyclin E and cdk5·GST-p25 to determine the mechanism of kinase inhibition by this compound. The Lineweaver-Burk plots of 1/*v* *versus* 1/[ATP] for PNU 112455A inhibition of both kinases converge on the *y* axis, demonstrating that the compound was competitive with respect to ATP (Fig. 3, *B* and *C*). Plots from experiments in which peptide substrate was varied demonstrated that PNU 112455A was a non-competitive inhibitor with respect to peptide (Fig. 3, *D* and *E*) for each of the enzymes.

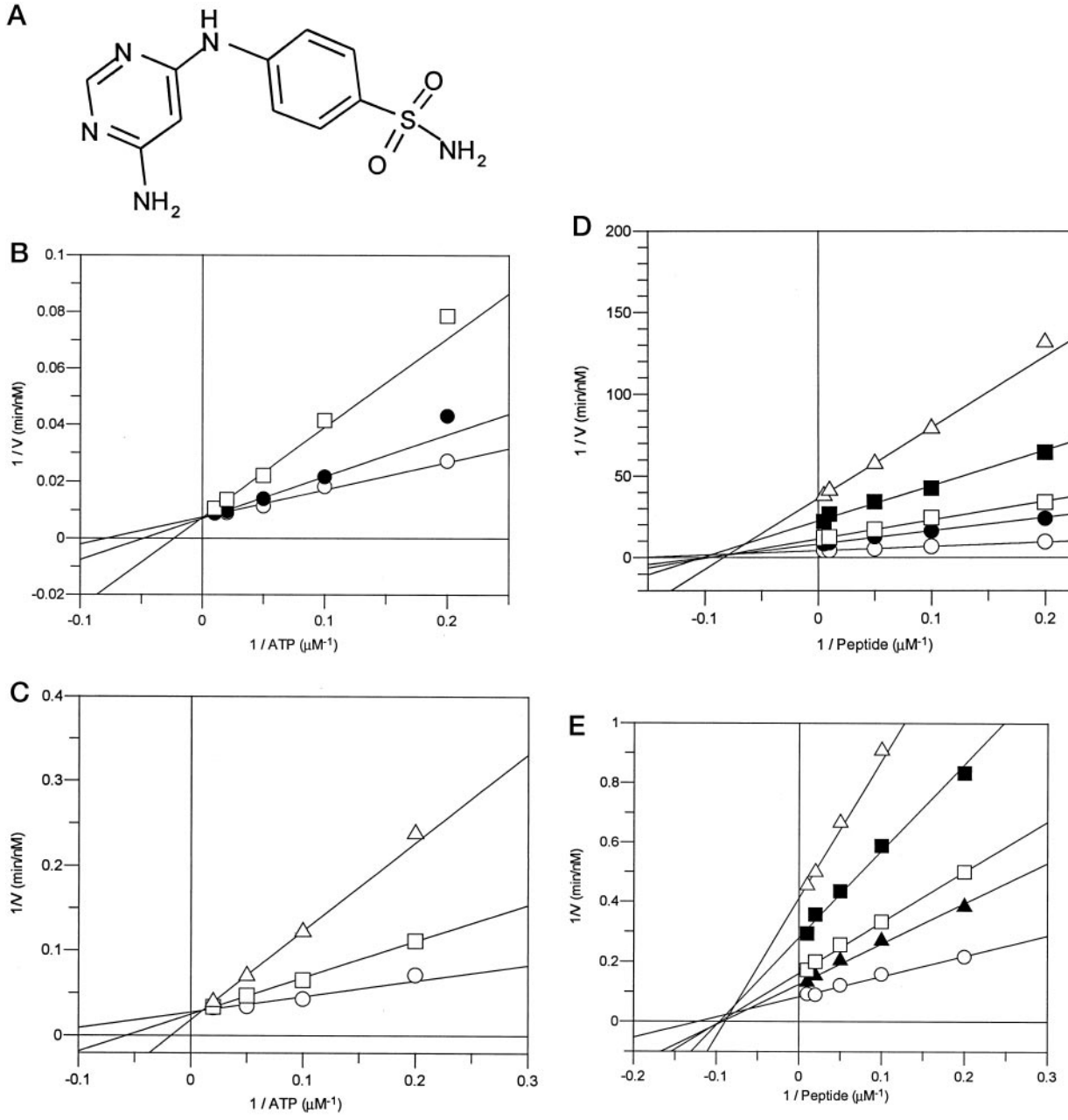

FIG. 3. **PNU 112455A is an ATP-competitive cdk inhibitor.** *A*, chemical structure of PNU 112455A. *B* and *C*, Lineweaver-Burk plots of PNU 112455A *versus* ATP with cdk2·GST-cyclin E (*B*) and cdk5·GST-p25 (*C*). *D* and *E*, Lineweaver-Burk plots of PNU 112455A *versus* peptide substrate with cdk2·GST-cyclin E (*D*) and cdk5·GST-p25 (*E*). PNU 112455A concentrations: ○, 0 μM; ●, 1 μM; ▲, 2.5 μM; □, 5 μM; ■, 10 μM; △, 20 μM.

Crystals of cdk2 were soaked with PNU 112455A, and Fig. 4*A* shows a ribbon drawing of the least-squares superimposed cdk2 crystal structures containing the natural substrate, ATP, and the inhibitor PNU 112455A. The inhibitor is located in the same aromatic favoring position as the adenine of the ATP·cdk2 structure and of many other inhibitors in co-crystal structures with cdk2 (35, 48). Fig. 4*B* shows that it also forms hydrogen bonds to residues Glu^{81} and Leu^{83} of cdk2, consistent with other co-crystal structures and the adenine moiety of ATP and substrate, thus providing direct structural detail of the ATP-competitive nature of the inhibitor.

PKV is a peptide-based inhibitor that corresponds to the peptide substrate with the substitution of valine for the phosphoacceptor threonine. Experiments were carried out with cdk5·GST-p25 and peptide substrate, in the presence of varying amounts of the inhibitor (Fig. 5*A*). As expected, Lineweaver-Burk plots of this data demonstrated that inhibition by the PKV peptide was competitive with peptide substrate with a K_i value of 0.6 ± 0.3 mM. The same competitive inhibition and K_i value were observed using cdk2·GST-cyclin E (data not shown). In contrast, in experiments with either kinase in which the concentration of ATP was varied, the PKV peptide was a non-competitive inhibitor of ATP (Fig. 5, *B* and *C*). Taken together, the results with PNU 112455A showing non-competitive inhibition with respect to peptide substrate, as well as the PKV inhibitor results, fulfill the criteria for demonstrating that cdk2 and cdk5 both utilize a random kinetic pathway (47, 49).

Table II lists the dissociation constants for ATP and peptide for both cdk2·GST-cyclin E and cdk5·GST-p25 calculated by simultaneous fits of the random equation to the data. K_a and αK_a are the dissociation constants for ATP in the absence and presence, respectively, of the peptide substrate in the kinase active site. Similarly, K_b and αK_b are the dissociation constants for peptide in the absence and presence of ATP in the active

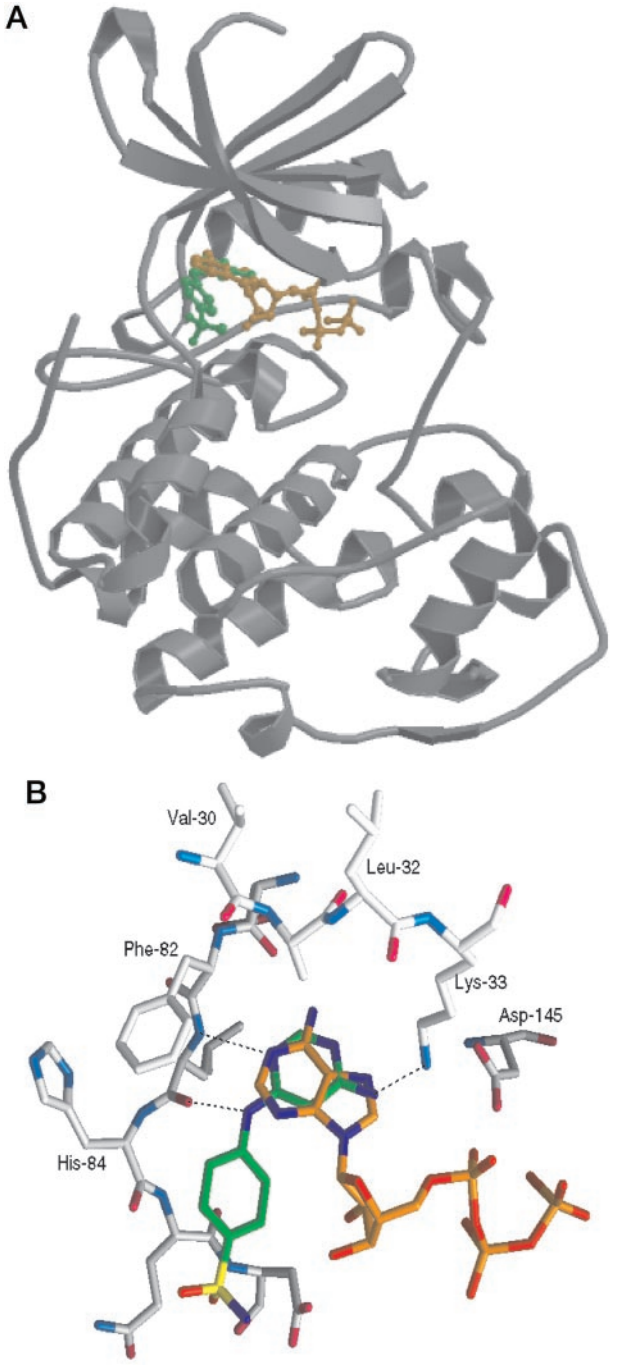

FIG. 4. **Crystal structure of PNU 112455A bound to cdk2.** *A*, schematic tracing of the cdk2 backbone shown with ATP (*brown*) (35) and PNU 112455A (*green*). Software in Refs. 63 and 64 was used to generate the figure. *B*, a close-up view of the ligand and the surrounding cdk2 environment, showing hydrogen bonds (*dotted lines*) made by PNU 112455A (*green* and *heteroatom colors*), and the relationship of this ligand to the ATP binding site determined in Refs. 35 and 46 (*orange* and *heteroatom colors*).

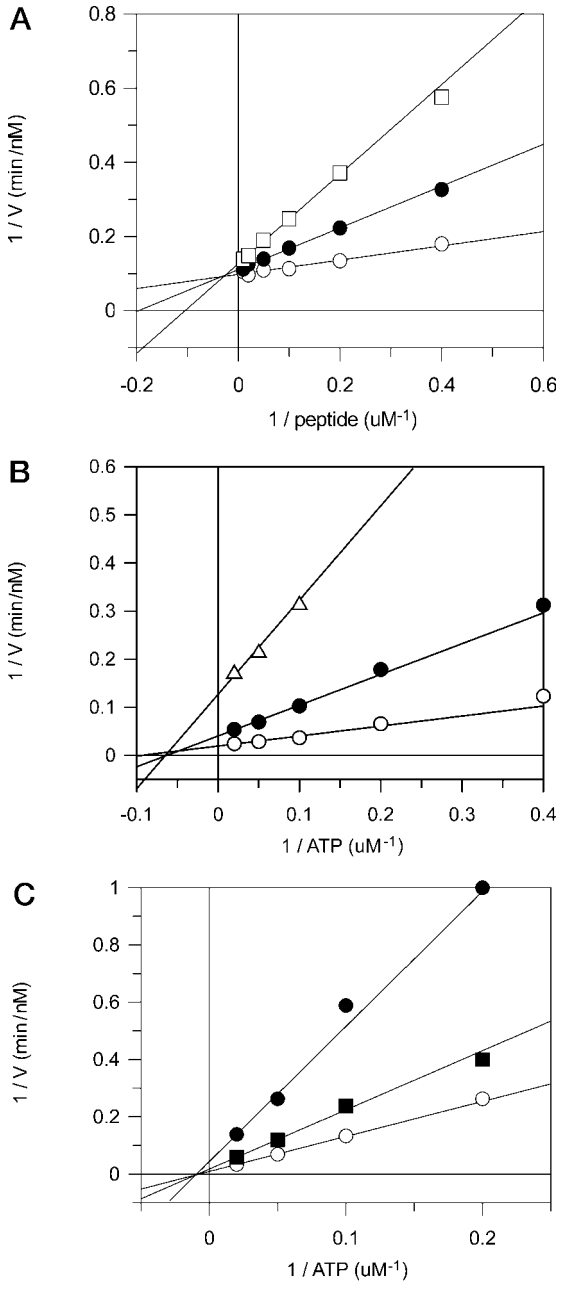

FIG. 5. **PKV is a competitive cdk inhibitor.** *A*, Lineweaver-Burk plot of PKV *versus* peptide substrate with cdk5·GST-p25. *B* and *C*, Lineweaver-Burk plots of PKV *versus* ATP with cdk2·GST-cyclin E (*B*) and cdk5·GST-p25 (*C*). PKV concentrations: ○, 0 mM; ■, 1 mM; ●, 2 mM; □, 4 mM; Δ 5 mM.

TABLE II
Summary of kinetic constants

Data are the average ± S.D. Data for cdk5·GST-p25 are from two experiments and from five experiments for cdk2·GST-cyclin E.

Parameter	cdk5·GST-p25	cdk2·GST-cyclin E
k_{cat}, s^{-1}	5.7 ± 0.2	15.1 ± 5.5
K_a, μM	3.2 ± 0.7	3.6 ± 1.0
K_b, μM	1.6 ± 0.3	4.6 ± 1.4
α	7.2 ± 1.8	130 ± 44

site, respectively. The K_a for both enzymes is similar, ~3 μM, which is comparable to that observed for other kinases. The K_b value, the peptide dissociation constant, is ~4 μM for cdk2·GST-cyclin E and ~2 μM for cdk5·GST-p25. The cooperativity factor, α, is greater than 1 for both enzymes, demonstrating that the binding of one substrate decreases the affinity for the second substrate. The degree of anticooperativity for cdk2·GST-cyclin E was large, with α greater than 100, whereas for cdk5 the value for α was moderate.

DISCUSSION

We have investigated the kinetic mechanism of two members of the cyclin-dependent kinase family, cdk2 complexed with cyclin E, and cdk5 complexed with p25. There is increasing interest in these enzymes as therapeutic targets, and thus

establishing their mechanism is an important step in the identification of inhibitors. Kinetic analysis has not been reported previously for either of these kinases, and we demonstrate that they utilize a random mechanism for ATP and peptide substrate binding.

Kinetic mechanisms have been investigated for a number of other kinases, and for most of them a sequential, and not a ping-pong, mechanistic pathway has been reported. For cAMP-dependent kinase Whitehouse *et al.* (50, 51) reported that the mechanism was ordered, with the nucleotide binding first while Cook *et al.* (30) reported that MgATP and peptide bind randomly, although initial binding of the nucleotide is preferred. An ordered sequential mechanism has been reported for p38 MAPK (32), for the vascular endothelial growth factor receptor-2 tyrosine kinase (52) and for the v-Src kinase (53). Both an ordered (54) and a random pathway (55) have been reported for the EGF receptor tyrosine kinase. A number of other kinases, including MEK and IκB, have been shown to utilize a random mechanism (31, 56, 57). In comparing these results, it is important to note that some of these studies have used a peptide as substrate, as in the present experiments, whereas others have used a full-length protein. Use of a substrate with a single phosphorylation site simplifies the kinetic analysis, however, it is possible that the use of a small peptide, compared with a physiological protein substrate, may affect the results.

We used two inhibitors to show the random mechanism for cdk2 and cdk5. The PKV peptide was competitive with respect to peptide substrate, whereas PNU 112455A competed with ATP. This compound is equipotent toward both kinases, with a K_i of ~2 μM. The crystal structure of PNU 112455A bound to cdk2 showed that the compound binds in the ATP binding site of the enzyme, with the aminopyrimidine ring oriented in a similar position as the adenine. Crystal structures of either cdk5 alone or of a complex of the compound with cdk5 have not been determined, however, there is a very high degree of sequence identity between cdk2 and cdk5 in the region that binds ATP and the ATP competitive inhibitors. This allows one to predict a reliable model of the three-dimensional structure of cdk5 in this region (58). Thus, PNU 112455A is expected to bind to cdk5 in the same orientation.

Among the cdk family members, a kinetic mechanism for cdk4·cyclin D1 was investigated using a peptide derived from the retinoblastoma (Rb) protein as substrate. Using staurosporine as a dead-end inhibitor, it was suggested that ATP binds first followed by the Rb peptide (59). It might be expected that all of the cdks would utilize the same kinetic pathway. However, cdk4 differs from the other cdk family members in several respects. For example, olomoucine and roscovitine potently inhibit cdk2 and cdk5 but show little or no inhibition of cdk4. In addition, cdk4 has a narrow substrate specificity compared with cdk2 and cdk5 (28). Differences in the kinetic mechanisms may contribute to these differences in substrate specificity and inhibitor sensitivity between cdk4 and cdk2 and cdk5.

Defining the ways enzyme activity is controlled is an important step toward understanding the roles of cdk2 and cdk5 *in vivo*. Multiple mechanisms of regulation have been reported for these kinases. In the case of cdk2, activity is regulated via phosphorylation of key residues (Thr^{14}, Tyr^{15}, and Thr^{160}) as well as by the availability of cyclins throughout the cell cycle. In addition, the endogenous inhibitory proteins (KIPs) contribute to the overall level of kinase activity (5, 60). Less is known about the regulation of cdk5 activity. Phosphorylation of the T loop is not required for cdk5 kinase activity, and endogenous inhibitory proteins have not been characterized (16, 61). The availability of p35 or p25 protein appears to be an important regulator of activity, and recent evidence suggests that the processing of p35 to p25 may result in increased kinase activity (25).

An interesting aspect of the kinetic analysis of cdk2·GST-cyclin E and cdk5·GST-p25 is the demonstration of anticooperativity (α) between the two substrates, which suggests another level of regulation. The α factor values were greater than one for both enzymes, although there was much greater anticooperativity for cdk2, compared with cdk5. These values indicate that binding of the first substrate (either ATP or peptide) greatly increases the effective K_m of the second substrate. For example, in the case of cdk2·GST-cyclin E, if the concentration of ATP was very low and held constant, then the apparent K_m for the peptide substrate would approach a minimum of 4.6 μM. At high ATP concentrations, such as 1 mM, the apparent K_m value for the peptide substrate would be increased almost 100-fold. Conversely, changes in the peptide substrate concentration would have a similar effect on the apparent K_m for ATP. This anticooperativity may play an important role in regulating cdk enzyme activity *in vivo*, because it implies that the enzymatic activity, without reaching saturation, is spread out over a very large substrate concentration range. Our results indicate that the anticooperativity of binding of the substrates may represent another mechanism by which tight control is maintained over the activation state of cdk2 and cdk5.

Values for α have been calculated for several other kinases acting via a random kinetic mechanism. For MEK, p38–2 MAPK, and Csk, an α factor of 1 has been reported (31, 56, 62). These results indicate that, for these kinases, binding of the first substrate does not influence binding of the second substrate. In contrast, for IκB kinase, the value of α is 0.11, indicating that the two substrates bind in a cooperative manner (57). Posner *et al.* (55) analyzed the kinetic mechanism of the EGF receptor tyrosine kinase and showed that for the unactivated receptor, α equals 20, demonstrating anticooperativity. In contrast, for the EGF-activated receptor kinase, α was markedly reduced, with a value of ~1. The authors suggest that EGF binding to the receptor induces conformational changes, which influence the binding of the substrates to the kinase (55). These results suggest that the activation state of the kinase is an important determinant in the degree of cooperativity of substrate binding. In the case of our cdk2·cyclin E experiments, the complex was purified from High-Five insect cells, which contain endogenous cdk-activating enzyme activity, resulting in an enzymatically active complex. It is not known whether this activation accurately mimics the conformational changes that occur upon activation in mammalian cells, but it could influence the value of α in our experiments. An additional factor, which may influence substrate cooperativity, is the substrate used in the assay. In our experiments a histone-derived peptide was used as the substrate. Further experiments are necessary to determine whether use of a more physiological protein substrate, such as the retinoblastoma protein for cdk2·cyclin E or the cytoskeletal protein tau for cdk5·p25, influences the anticooperative nature of the substrate binding.

Acknowledgments—The helpful suggestions and analyses of the kinetic data by Dr. Ferenc Kezdy and the assistance of Dr. Barry Finzel with the crystallography figures are gratefully acknowledged.

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